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Development of an Enzyme-Linked Immunosorbent Assay To Detect an Immunomodulatory α-D-Glucan–Protein Complex, MPG-1, in Basidiomycete *Tricholoma matsutake* and Related Processed Foods

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We previously isolated a novel immunomodulatory α-(1,4)(1,6)(1,2)-D-glucan–protein complex (MPG-1) from mycelia of *Tricholoma matsutake* in basidiomycetes. In the present study, we raised a polyclonal antibody by immunizing rabbits with MPG-1 and constructed a sandwich enzyme-linked immunosorbent assay (ELISA) system to examine the distribution of MPG-1 among edible mushrooms and related processed foods. The system detected MPG-1 quantitatively at concentrations of more than 10 ng/mL, with a coefficient of variation of less than 10% by intra-assay and interassay precision. Analysis with the system of chemically modified MPG-1 suggested that the sugar moiety was mainly involved in the detection. The system detected MPG-1 in the extracts of the fruiting bodies of *T. matsutake* but not in those of 34 other basidiomycete species. Moreover, a significant amount of MPG-1 was detected in the extracts of their cultured mycelia. The antigenic structure of MPG-1 was relatively stable in terms of pH and temperature. MPG-1 was detected in processed foods supplemented with *T. matsutake*. These results suggest that MPG-1 is distributed predominantly in *T. matsutake* species and that the ELISA system can detect it in processed foods supplemented with *T. matsutake*.

KEYWORDS: *Tricholoma matsutake*; immunomodulatory α-D-glucan–protein complex; MPG-1; α-D-glucan; enzyme-linked immunosorbent assay (ELISA)

INTRODUCTION

Glucans are a structurally diverse group of polysaccharides with D-glucopyranosyl units involved in various physiological functions and distributed in a wide variety of sources such as barley, oats, algae, bacteria, yeasts, fungi, and mushrooms. Of the two types of glucan anomer, β-(1,4)-D-glucans such as cellulose play important roles as a component of dietary fibers that modulate intestinal environments and as a dispersant stabilizer in processed food or medical products (*1*). Linear β-(1,3)-D-glucans with either β-(1,6)- or β-(1,4)-D-glucan side chains exhibit antitumor activities, anti-infectious activities, immunomodulating activities, or blood cholesterol-lowering activities (*2, 3*) and are used as supplements or medicines. Methods for the analysis of β-D-glucans have been developed and applied to the food industry, brewing industry, and health care industry. They include measurements of the intense fluorescence produced by a complex of aniline blue dye with β-D-glucan (*4*), spectrophotometric measurements of liberated reducing sugars produced by the enzymatic degradation of β-D-glucan (*5*), measurements of β-D-glucan by the Limulus amebocyte lysate (LAL) assay (*6*), and an enzyme-linked immunosorbent assay (ELISA) using specific antibodies (*7, 8*).

The other type of glucan anomer, α-D-glucan, plays important roles mainly in energy sources. Starch is composed of two distinct polymers: amylose, which is a linear chain of α-(1,4)-linked glucose units, and amylopectin, which consists of linear chains of α-(1,4)-linked glucose units with extensive branching resulting from a α-(1,6)-linkage per 24–30 glucose residues (*9*). Glycogen structurally resembles starch but is more extensively branched, with a branch every 8–10 residues. Although the nutritional qualities of glycogen and its metabolites have been investigated, mainly in individuals under dietary restriction or exercise (*10*), few papers have reported on biological activities of α-D-glucans; administration of α-D-glucan or α-D-1,6 and 1,4 glucan complex isolated from basidiomycete *Agaricus blazei*

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suppressed the growth of sarcoma 180 in mice (*11*, *12*). Moreover, intratumor inoculation of the acid-treated α-D-glucan fraction from *A. blazei* induced apoptosis in Meth-A fibrosarcoma and suppressed growth in mice (*13*). Gelatinization and liberated reducing sugars produced by hydrolysis with α-amylase or hydrochloric acid (*14*) have been employed to measure α-D-glucans in the food and other industries.

In the process of searching for edible mushrooms and herbs that modulate immune responses when administered orally, we found that cultured mycelia of *Tricholoma matsutake* strain BP-7304, a member of the Tricholomataceae family of basidiomycetes, enhanced the recovery of natural killer cell activity that had been reduced by the loading of restraint stress (*15*, *16*). We purified a sodium hydroxide extract of the mycelia by a combination of ion-exchange chromatography and gel filtration to identify the components involved in the activity and obtained a single peak fraction (MPG-1) by reversed-phase chromatography (*17*). MPG-1 was an α-D-glucan–protein complex with a molecular mass of about 360 kDa. The sugar chain structure was an α-(1,4)-D-glucan linkage with α-(1,6)-D- and α-(1,2)-D-glucan linkages, and the amino acid composition included glutamine, alanine, asparagine, leucine, glycine, valine, serine, threonine, isoleucine, and proline.

The oral administration of MPG-1 promoted, in a dose-dependent manner, the recovery of the natural killer cell activity and serum IL-12 level in restraint-stressed mice (*17*). Moreover, the oral administration of the extracts of the fruiting bodies of *A. blazei*, *Lentinula edodes*, and *Grifola frondosa* in basidiomycetes, which are composed mainly of β-D-glucans as active agents, hardly caused any significant acceleration of the recovery of activity in restraint-stressed mice (*15*). The results suggest that the administration of MPG-1 may modulate immune responsiveness of individuals, possibly by a different manner from β-D-glucans.

There are components common to both fruiting bodies and mycelia, and components specifically exist in fruiting bodies or mycelia in basidiomycetes. It is important in terms of material supply to investigate whether MPG-1 exists only in *T. matsutake* mycelia or also in the *T. matsutake* fruiting bodies or other edible mushrooms; however, there are limitations in screening MPG-1-producing mushrooms from medicinal or edible mushrooms. In our preliminary study, we purified a fraction that was mainly composed of α-D-glucan and protein from the *T. matsutake* fruiting bodies and confirmed that its immunomodulative activity was almost the same as MPG-1 from mycelia (data not shown). However, conventional analysis of sugars such as the Association of Official Analytical Chemists (AOAC) International (*18*) and microelectrophoresis (*19*) could not distinguish MPG-1 from other glucans without activity.

ELISA is not only a measuring method with excellent sensitivity or selectivity but is also a relatively simple procedure able to measure multisamples in some cases (*7*). So, to further characterize the nature of MPG-1, we raised a polyclonal antibody (pAb) against MPG-1 and established a sandwich ELISA system to detect MPG-1. In the present study, we analyzed *T. matsutake* mycelia or 34 species of fruiting bodies and 16 species of mycelia in basidiomycetes for MPG-1 and used the ELISA system to detect its antigenic structure in processed foods.

MATERIALS AND METHODS

Preparation of MPG-1. MPG-1 was prepared according to the method of Hoshi et al. (*17*). Briefly, 100 g of dried powder of cultured mycelia of *T. matsutake* strain BP-7304 (Kureha Corp., Tokyo, Japan) was added to a 0.2 M sodium hydroxide solution, stirred for 1 h at 25 °C, and centrifuged at 15000*g* for 20 min. The supernatant was collected and added to an equal volume of a 1:1 mixture of chloroform and methyl alcohol for fractionation to recover the water-extractable fraction (EW). Next, fraction EW was sequentially fractionated by DEAE ion-exchange chromatography and gel filtration chromatography to recover MPG-1. MPG-1 showed a single peak and band on reversed-phase high-performance liquid chromatography (HPLC) and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), respectively. Physicochemical properties such as sugar components, amino acid components, NMR, and IR coincided well with those described by Hoshi et al. (*17*). In addition, a hydrazine-modified MPG-1 (protein-free MPG-1) and a periodate-modified MPG-1 [α-(1,4)(1,6)(1,2)-D-glucan-free MPG-1] were prepared as described by Matsunaga et al. (*20*) and Takasaki et al. (*21*), respectively.

Immunization of Rabbits with MPG-1 and Determination of Antiserum Titer. Five hundred micrograms of MPG-1 was dissolved in 0.5 mL of phosphate-buffered saline (PBS) and emulsified with an equal volume of Freund's complete adjuvant (Sigma-Aldrich Co., Tokyo, Japan). The emulsion was injected subcutaneously at 10 different sites on the backs of female New Zealand white rabbits (Charles River Japan, Inc., Kanagawa, Japan). Two weeks after the first immunization, the emulsion of MPG-1 with Freund's incomplete adjuvant (Sigma-Aldrich Co.) was injected subcutaneously four times at 2 week intervals, followed by a mixture of MPG-1 with TiterMax gold adjuvant (Interchim, Montluçon, France) four times at 2 week intervals. During the immunization period, about 1 mL of blood was drawn from an ear vein for the determination of titers. Seven days after the final immunization, rabbits were anesthetized with a xylazine/ketamine cocktail, and blood was collected via cardiac puncture. Serum samples were separated and stored at −80 °C until used.

The titer of the antiserum was determined as follows: Each well of a Costar E.I.A. R.I.A. eight well strip 96 well microtiter plate (Corning Inc., Tokyo, Japan) was coated with 1 μg/mL of MPG-1 in 100 μL of coating buffer (0.05 M sodium carbonate and 0.05 M sodium hydrogen carbonate in pure water, pH 9.6) and incubated at room temperature (RT) for 3 h. Each well was washed with PBS and blocked with 1% bovine serum albumin (BSA) in PBS at RT for 30 min to prevent nonspecific protein binding. After further washing of each well with PBS containing 0.05% Tween 20 (PBS-T), antiserum (1:100–1:24300 dilution; 100 μL/well) was added, and the mixture was incubated at RT for 3 h. After the wells were washed with PBS-T, alkaline-phosphatase (AP)-conjugated goat antirabbit IgG (1:3000, 100 μL/well) (KPL Inc., Gaithersburg, MD) was added to each well and incubated at RT for 1 h. After more washing, 100 μL of 5-bromo-4-chloro-3-indoxyl phosphate solution (KPL Inc.) was added. Color development was stopped by the addition of 100 μL of a 2.5% ethylenediaminetetraacetic acid solution (KPL Inc.), and the optical density at 630 nm of each sample was measured.

Purification of Antiserum. Antiserum was diluted with 20 mM sodium phosphate (pH 7.0) and applied to a column packed with recombinant protein A Sepharose (GE Healthcare UK Ltd., Little Chalfont, United Kingdom), according to the manufacturer's instructions. After the column was washed with 20 mM sodium phosphate, the IgG fraction (pAb) was eluted with 0.1 M glycine-hydrochloric acid (pH 2.7), which was immediately neutralized with 10% (v/v) 1 M Tris-HCl buffer (pH 8.0). The solvent was replaced by PBS using a Sephadex G-25 Superfine column (GE Healthcare UK Ltd.).

Preparation of Biotin-Labeled A pAb. pAb was labeled using a Sulfo-OSu biotinylation kit (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. Briefly, to a tube containing 10 mg/mL of pAb in 50 mM sodium hydrogen carbonate buffer, 10 mg/750 μL of Biotin-$(AC_5)_2$Sulfo-OSu solution was added, and the mixture was allowed to react at RT for 2 h. Then, the reaction mixture was applied to a gel filtration column, and a biotin-labeled antibody solution was recovered.

Sandwich ELISA. The ELISA was performed as follows. Wells of a 96 well microtiter plate (Corning Inc., United States) were coated with 5 μg/mL of antibody in 100 μL of 0.1 M carbonate buffer (pH 9.5) and kept at 4 °C overnight. The plate was washed with PBS, and 1% BSA in PBS was added for 30 min at RT. Next, 100 μL of the

sample or MPG-1 solution was added to each well and incubated at RT for 2 h. After each well was washed with PBS-T, biotinylated pAb (1 μg/mL, 100 μL/well) was added, and the mixture was incubated at RT for 2 h. After the mixture was further washed with PBS-T, horseradish peroxidase (HRP)-conjugated streptavidin (0.1 μg/mL, 100 μL/well) (Zymed Laboratories, Inc., South San Francisco, CA) was added to the wells, and the mixture was incubated at RT for 1 h. After more washing, 100 μL of glycine-citric acid buffer containing 2,2′-azino-di-[3-ethylbenzthiazoline sulfonate] and hydrogen peroxide (KPL Inc.) was added to each well. Color development was stopped by the addition of 100 μL of a 5% SDS solution (ABTS Peroxidase Stop Solution, KPL Inc.), and the optical density was measured at 405–630 nm. The concentration of MPG-1 in each sample was estimated by extrapolating the optical density into a calibration curve.

Glucans. Glucans with an α-anomeric structure (oyster glycogen, bovine liver glycogen, rice starch, wheat starch, corn starch, potato amylose, amylopectin from potato starch, pullulan from *Aureobasidium pullulans*, and corn dextrin) and glucans with a β-anomeric structure (laminarin from *Laminaria digitata*, yeast glucan from *Saccharomyces cerevisiae*, and barley glucan from *Hordeum vulgare*) were purchased from Sigma-Aldrich Co. (Tokyo, Japan). Lentinan and schizophyllan were purchased from Ajinomoto Co. Ltd. (Tokyo, Japan) and Kaken Pharmaceuticals. Co. Ltd. (Tokyo, Japan), respectively. The α-D-Glucan fraction (FA-1-a-α) and β-D-glucan fraction (FA-1-a-β) were purified from a hot water extract of the fruiting bodies of *A. blazei* by ethanol precipitation followed by ion-exchange chromatography and gel filtration, according to the method described by Mizuno et al. (*11*). Acidic proteo-heteroglycan, PL, was purified from a hot water extract of the fruiting bodies of *Phellinus linteus* by ethanol precipitation followed by ion-exchange chromatography, according to the method described by Kim et al. (*22*). The physicochemical properties and structures identified using these references were examined in terms of sugar and protein contents by the phenol-sulfuric acid method and the copper-Folin method. The constitutive sugar components were identified by HPLC with postcolumn derivatization, and the mode of sugar chain linkage was identified by NMR and methylation analysis (*17*). It was confirmed that the main structure of FA-1-a-α was α-(1,4)(1,6)-D-glucan, and that of FA-1-a-β was β-(1,3)(1,6)-D-glucan. PL was composed of α-D-glucan and β-D-glucan, the main constitutive sugars of which were glucose, mannose, and galactose.

Preparation of Hot Water Extracts of Fruiting Bodies. Fruiting bodies of edible mushrooms purchased at a market in Japan in autumn 2004 were identified at the species level morphologically by mycologists in our laboratories (*23*). After the fruiting bodies were lyophilized and powderized, 1.0 L of pure water was added to 20 g of the powder, extracted in a water bath at 98 °C for 3 h, and centrifuged at 15000*g* for 20 min at 4 °C. The supernatant was collected, dialyzed against pure water, and lyophilized. These samples were stored at 4 °C until used. To avoid variation in preparations, 10–15 pieces of fruiting bodies (500 g or more in wet weight) were mixed and used as one sample in the experiments.

Preparation of Hot Water Extracts of Cultured Mycelia. Mycelia of mushrooms (*T. matsutake* strain BP-7304, *Tricholoma flavovirens* strain CM631-2, *Armillariella mellea* strain CM533-7, *Flammulina verutipes* strain CM602-4, *L. edodes* strains CM587-1 and CM587-3, *Lyophyllum shimeji* strain CM502-5, *Lyophyllum ulmarium* strain CM507-1, *Agaricus bisporous* strain CM747-2, *Auricularia auricula* strain CM728-4, *Pleurotus cornucopiae* strain CM577-1, *Pleurotus ostreatus* strains CM567-2 and CM567-8, *Pleurotus pulmonaris* strain CM573-1, *G. frondosa* strain CM756-2, *Schizophyllum commune* strain CM577-2, *Hypholoma sublateritium* strain CM691-2, *Pholiota nameko* strain CM887-2, and *Pholiota squarrosa* strain CM235-3) maintained in our laboratory by serial passage in slant agar were used in the study. Mycelia of *T. matsutake* strains NBRC06915, NBRC06925, NBRC06930, NBRC06935, NBRC30604, NBRC30605, and NBRC30606 were transferred from the National Institute of Technology and Evaluation (NITE) Biological Resource Center (Chiba, Japan), and MAFF460038 was transferred from the National Institute of Agrobiological Sciences (NIAS) Genebank (Ibaraki, Japan).

These mycelia were inoculated in the culture medium (3% glucose and 0.3% yeast extract in pure water, pH 6.0) and cultured at 24 °C for 14–35 days. After cultivation, the mycelia were collected, washed with pure water, lyophilized, and powderized. The hot water extraction procedure was the same as that for the fruiting bodies.

Stability of MPG-1 in *T. matsutake* Mycelia and Fruiting Bodies. For the evaluation of pH stability in suspension, 5 g of dried powder of *T. matsutake* fruiting bodies sampled at Kyoto or strain BP-7304 mycelia was suspended in 100 mL of citrate buffer at pH 3.0, 4.0, or 5.0 or phosphate buffer at pH 6.0, 7.0, or 8.0 and kept at 25 °C for 3 h. For the evaluation of temperature stability in suspension, 5 g of dried powder of the *T. matsutake* fruiting bodies or mycelia was suspended in 100 mL of PBS (pH 7.0) and kept in a water bath at 40, 60, or 98 °C for 3 h or in an autoclave at 121 °C for 15 min. After the treatment, the suspension was homogenized. This was followed by extraction in a water bath at 98 °C for 3 h and centrifugation at 15000*g* for 20 min at 4 °C. The supernatant was collected for the determination of MPG-1 content.

For the evaluation of temperature stability in a powdered form, 5 g of dried powder of the *T. matsutake* fruiting bodies or mycelia was kept in an oil bath at 40, 60, 100, or 150 °C for 0.5, 1, 2, 4, or 8 h under a nitrogen atmosphere. After the treatment, the powder was suspended in 100 mL of pure water and homogenized. This was followed by extraction in a water bath at 98 °C for 3 h and centrifugation at 15000*g* for 20 min at 4 °C. The supernatant was subjected to dialysis against pure water at 4 °C for 48 h, followed by freeze-drying.

Preparation of Processed Foods, Soups, and Tea. Dried soup powder supplemented with the *T. matsutake* fruiting bodies (Marumiya Corp., Tokyo, Japan) and dried soup powder supplemented with the *L. edodes* fruiting bodies (Nagatanien Co. Ltd., Tokyo, Japan) were purchased at a market, and soups were prepared according to the manufacturer's directions. Retort-packed *T. matsutake* fruiting bodies (Ezaki Glico Co., Ltd., Osaka, Japan) and retort-packed *A. bisporus* and *P. ostreatus* fruiting bodies (Ezaki Glico Co., Ltd.) were purchased in a market, and boiled rice was prepared according to the manufacturer's directions. Furthermore, boiled rice supplemented with *T. matsutake* mycelia was prepared as follows: 630 mL of pure water, 420 g of rice, and 1.8 g of dried powder of strain BP-7304 mycelia were mixed and boiled in a SR-VTM10 rice cooker (Matsushita Electric Ind. Co. Ltd., Osaka, Japan), according to the manufacturer's instructions. Bread supplemented with *T. matsutake* was prepared as follows: 210 mL of pure water, 330 g of wheat flour, 3 g of dried baker's yeast, and 1.8 g of dried powder of strain BP-7304 mycelia were mixed well and fermented and then baked for 15 min at 200 °C in an oven. Cookies supplemented with *T. matsutake* were prepared as follows: 330 g of wheat flour, 100 g of butter, 80 g of sugar, two eggs, and 1.8 g of dried powder of strain BP-7304 mycelia were mixed well and baked for 15 min at 150 °C in an oven. Tea supplemented with *T. matsutake* was prepared as follows: 6 g of tea and 1.8 g of dried powder of strain BP-7304 mycelia were added to a teapot, into which hot distilled water was poured. Yogurt supplemented with *T. matsutake* was prepared as follows: 440 mL of milk, 1.8 g of strain BP-7304 mycelia, and 60 mL of a commercially available plain yogurt as a starter were agitated and made to ferment by a MM-AV05D yogurt-maker (Matsushita Electric Ind. Co. Ltd.) for 8 h, according to the manufacturer's instructions.

Soups and teas were centrifuged at 15000*g* for 20 min at 4 °C, and the supernatants were stored at −80 °C until used. Three liters of pure water was added to 30 g of the above-processed foods. These mixtures were homogenized by the use of a homogenizer and extracted for 3 h in a water bath at 98 °C. Then, the homogenates were centrifuged at 15000*g* for 20 min at 4 °C, and the supernatant was subjected to dialysis against pure water at 4 °C for 48 h, followed by freeze drying.

RESULTS AND DISCUSSION

Establishment of a Sandwich ELISA System To Detect MPG-1. Female New Zealand white rabbits were subcutaneously immunized with MPG-1 at 2 week intervals, and the titer of sera was periodically monitored by ELISA. Although the titer only increased slowly until the sixth immunization, it increased markedly after the seventh immunization, reaching a plateau after the eighth immunization. **Figure 1** shows a titration

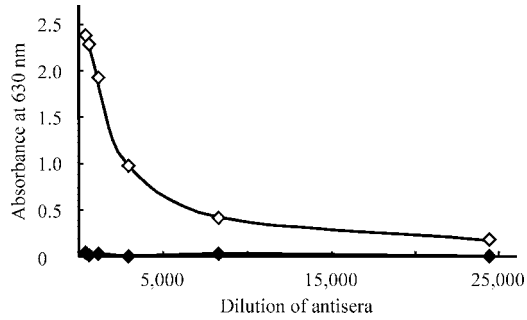

Figure 1. Titration curve of rabbit anti-MPG-1 serum after the ninth immunization: ◇, antiserum 7 days after the ninth immunization; ♦, nonimmune serum. One hundred microliters of antiserum 7 days after the ninth immunization (1:100–1:24300 diluted) was dispensed into each MPG-1-coated well and incubated at RT for 2 h. The titer of the antiserum was determined by ELISA as described in the Materials and Methods. The longitudinal axis and horizontal axis represent absorbance at 630 nm (in linear scale) after subtraction of the background and dilution of the sera (in linear scale), respectively. Each value represents the mean of three independent determinations.

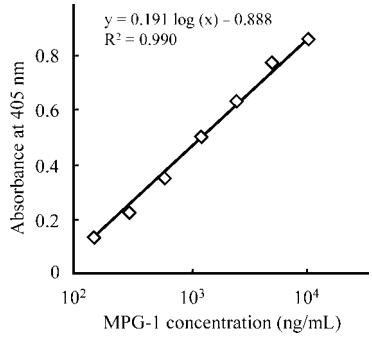

Figure 2. Relationship between concentration of MPG-1 and absorbance in sandwich ELISA. The longitudinal axis and horizontal axis represent absorbance at 405 nm (in linear scale) after subtraction of the background and MPG-1 concentration (in log scale), respectively. Each value represents the mean of three independent determinations.

curve of sera sampled after the ninth immunization. Absorbance at 630 nm was maximal at the centuple dilution of the sera, gradually decreased as the sera were diluted, and dropped below the detection level at a 24300-fold dilution.

Seven days after the ninth immunization, sera of rabbits were purified to obtain the IgG fraction (pAb) by protein A affinity chromatography, and a sandwich ELISA system was constructed using pAb-coated wells of a 96 well plate for capture, with biotin-labeled pAb as a detector, and HRP-labeled streptavidine as a probe. As shown in **Figure 2**, when MPG-1 at 5–2 $\times$ 10^4 ng/mL was applied to the ELISA system, there was a linear plot between 1.5 $\times$ 10^2 and 1 $\times$ 10^4 ng/mL, and the calibration curve showed the following formula: [$(y) = 0.191 \log(x) - 0.888$, $R^2 = 0.990$], where (y) is the concentration of MPG-1 and (x) is the absorbance at 405 nm. The detection limit with the system was 10 ng/mL of MPG-1.

To assess precision within an assay, each assay was performed five times at concentrations of 6 $\times$ 10^2, 2.5 $\times$ 10^3, and 1 $\times$ 10^4 ng/mL. Furthermore, to assess precision between assays, each assay was repeated five times at MPG-1 concentrations of 3 $\times$ 10^2, 6 $\times$ 10^2, 1.2 $\times$ 10^3, 2.5 $\times$ 10^3, 5 $\times$ 10^3, and 1 $\times$ 10^4 ng/mL. The coefficient of variation (standard deviation $\times$ 100/mean value) was 10% or less in all experiments, suggesting that the variation in measurements was minimal (data not shown).

Table 1. Reactivity of pAb to Various Polysaccharide Preparations As Measured by a Sandwich ELISA

glucans	major sugar units[a]	major sugar chain linkages	reactivity
MPG-1	α-D-Glc	-(1,4)(1,6)(1,2)-	+
hydrazine-modified MPG-1		-(1,4)(1,6)(1,2)-	+
periodate-modified MPG-1			NR[b]
glycogen (oyster)[c]	α-D-Glc	-(1,4)(1,6)-	NR
glycogen (bovine liver)		-(1,4)(1,6)-	NR
starch (rice)		-(1,4)(1,6)-	NR
starch (wheat)		-(1,4)(1,6)-	NR
starch (corn)		-(1,4)(1,6)-	NR
amylose (potato)		-(1,4)(1,6)-	NR
amylopectin (potato starch)		-(1,4)(1,6)-	NR
pullulan (*A. pullulans*)		-(1,4)(1,6)-	NR
dextrin (corn)		-(1,4)(1,6)-	NR
FA-1-a-α (*A. blazei*)		-(1,4)(1,6)-	NR
lentinan (*L. edodes*)	β-D-Glc	-(1,3)(1,6)-	NR
schizophyllan (*S. commune*)		-(1,3)(1,6)-	NR
laminarin (*L. digitata*)		-(1,3)(1,6)-	NR
yeast glucan (*S. cerevisiae*)		-(1,3)(1,6)-	NR
barley glucan (*H. vulgare*)		-(1,3)(1,6)-	NR
FA-1-a-β (*A. blazei*)		-(1,3)(1,6)-	NR
inulin (chicory)	β-D-Frc, Glc	-(1,2)-	NR
PL (*P. linteus*)	Man, Glc, Gal	α-, β-glycoside linkages	NR

[a] Glc, glucose; Frc, fructose; Man, mannose; and Gal, galactose. [b] NR (no reactivity) indicated that the value was below the threshold of the assay. [c] The origin of the polysaccharide is given in parentheses.

Specificity of the Sandwitch ELISA System. The specificity of the ELISA system was evaluated using chemically modified MPG-1 and reagents of α-D-glucans and β-D-glucans. The ELISA system detected the antigenic structure of a hydrazine-treated MPG-1; however, it hardly detected a periodate-treated MPG-1, suggesting that it detected the sugar moiety of MPG-1 (**Table 1**).

The specificity of the ELISA system was then examined using 10 α-D-glucans: glycogen (oyster or bovine liver origin), starch (rice, wheat, or corn origin), amylose, amylopectin, pullulan, dextrin, and FA-1a-α. However, the system detected none of these glucans (**Table 1**). Furthermore, the ELISA system did not detect any of the β-D-glucans (lentinan, schizophyllan, laminarin, yeast glucan, barley glucan, and FA-1-a-β), inulin, or proteoglycan PL. These results demonstrate that the ELISA system predominantly recognizes the sugar structure of the MPG-1 molecule sensitive to periodate treatment.

Distribution of MPG-1 in Fruiting Bodies or Mycelia of Various Basidiomycetes. To examine the distribution of MPG-1 among edible mushrooms, hot water extracts of fruiting bodies were applied to the ELISA system at a concentration of 10 mg/mL. As shown in **Table 2**, the MPG-1 structure was detected in 11 species of fruiting bodies of *T. matsutake*; however, it was below the detection level in 34 mushrooms that belong to other species in the genus *Tricholoma* or families other than Tricholomataceae in basidiomycetes. The levels of MPG-1 in the hot water extracts of *T. matsutake* differed among sampling districts, being highest at Kyoto, Japan (374 μg/g), and lowest at Hiroshima (85 μg/g).

Next, to confirm the distribution of MPG-1, hot water extracts of mycelia were subjected to analysis. The level of MPG-1 in *T. matsutake* strain BP-73043 was highest (764 μg/g), and levels in extracts for eight standard strains of *T. matsutake* were 112–192 μg/g (**Table 3**). However, MPG-1 was below the detection level in cultured mycelia of *T. flavovirens*, which belongs to the genus *Tricholoma*, and those which belong to genera other than *Tricholoma* or families other than Tricholomataceae.

Table 2. MPG-1 Contents of Hot Water Extracts of Edible or Medicinal Mushrooms As Measured by a Sandwich ELISA

family	species	sampling location	MPG-1 content (μg/g)[a]
Tricholomatacea	*T. matsutake*	Kyoto, Japan	374 ± 43
		Iwate, Japan	199 ± 40
		Nagano, Japan	183 ± 28
		Okayama, Japan	104 ± 16
		Hiroshima, Japan	85 ± 25
		Jilin, China	115 ± 12
		Shichuan, China	106 ± 19
		Yunnan, China	137 ± 22
		Kangwon-do, Korea	159 ± 35
		North Korea	95 ± 14
		Canada	104 ± 11
	Tricholoma auratum	Akita, Japan	ND[b]
	Tricholoma portentosum	Nagano, Japan	ND
	Tricholoma giganteum	Kumamoto, Japan	ND
	F. velutipes	Nagano, Japan	ND
	Hypsizygus marmoreus	Niigata, Japan	ND
	L. edodes	Ooita, Japan	ND
	Lepista sordida	Fukushima, Japan	ND
	Lyophyllum decastes	Hokkaido, Japan	ND
	L. shimeji	Nagano, Japan	ND
Agaricaceae	*A. bisporus*	Niigata, Japan	ND
	A. blazei	Brazil	ND
	Agaricus campestris	Ishikawa, Japan	ND
Clavicipitaceae	*Cordyceps sinensis*	China	ND
Climacodontaceae	*Mycoleptodonoides aitchisonii*	Gunma, Japan	ND
Hericiaceae	*Hericium erinaceum*	Gunma, Japan	ND
Hydnaceae	*Hydnum repandum*	Nagano, Japan	ND
Hymenochaetaceae	*Inonotus obliqua*	Nagano, Japan	ND
	P. linteus	Korea	ND
Pleurotaceae	*P. cornucopiae*	Hokkaido, Japan	ND
	Pleurotus eryngii	Nagano, Japan	ND
	P. ostreatus	Fukushima, Japan	ND
Polyporaceae	*Coriolus versicolor*	Tochigi, Japan	ND
	Ganoderma applanatum	China	ND
	Ganoderma lucidum	Ooita, Japan	ND
	G. frondosa	Nagano, Japan	ND
Schizophyllaceae	*S. commune*	Nagano, Japan	ND
Sparassidaceae	*Sparassis crispa*	Ibaraki, Japan	ND
Strophariaceae	*Hypholoma sublateritium*	Nagano, Japan	ND
		Fukushima, Japan	ND
	P. nameko	Gunma, Japan	ND
	P. squarrosa	Fukuoka, Japan	ND
	Stropharia rugosoannulata	Niigata, Japan	ND
Thelephoraceae	*Sarcodon aspratus*	Nagano, Japan	ND
	Sarcodon scabrosus	Nagano, Japan	ND

[a] Each value represents the mean ± SD of three independent determinations. Figures express MPG-1 content (μg)/hot water extract (g) in dried weight. [b] ND (not detected), below the threshold of the assay.

Stability of MPG-1 in Fruiting Bodies or Mycelial Preparation of *T. matsutake*. To examine the stability of MPG-1 during heating, a suspension of *T. matsutake* fruiting bodies or mycelial preparation, which has been recently in market and used as a functional foodstuff in Japan (*24*), was kept for 3 h in PBS at temperatures of 40, 60, or 98 °C or 15 min in an autoclave at 121 °C. As a result, the levels of MPG-1 in the extracts of all experimental groups were almost the same as that of the control group (25 °C) (**Table 4**). Moreover, a suspension of *T. matsutake* fruiting bodies or mycelial preparation in a buffer at pH 3.0, 4.0, 5.0, 6.0, 7.0, or 8.0 was kept for 3 h at 25 °C. Then, the levels of MPG-1 in the extracts of all experimental groups were 95–100, when setting the level for the pH 7.0 group to 100 (data not shown). These results show that the MPG-1 in a suspension was relatively stable below 40–98 °C for 3 h or 121 °C for 15 min or at pH 3.0–8.0 for 3 h.

Table 3. MPG-1 Contents of Hot Water Extracts from Cultured Mycelia of Various Basidiomycetes As Measured by a Sandwich ELISA

family	species	strain	MPG-1 content (μg/g)[a]
Tricholomatacea	*T. matsutake*	BP-7304	764
		NBRC6915	153
		NBRC6925	112
		NBRC6930	177
		NBRC6935	165
		NBRC30604	154
		NBRC30605	172
		NBRC30606	192
		MAFF460038	183
	T. flavovirens	CM631-2	ND[b]
	A. mellea	CM533-7	ND
	F. velutipes	CM602-4	ND
	L. edodes	CM587-1	ND
		CM587-3	ND
	L. shimeji	CM502-5	ND
	L. ulmarium	CM507-1	ND
Agaricaceae	*A. bisporus*	CM747-2	ND
Auriculariaceae	*A. auricula*	CM728-4	ND
Pleurotaceae	*P. cornucopiae*	CM577-1	ND
	P. ostreatus	CM567-2	ND
		CM567-8	ND
	P. pulmonaris	CM573-1	ND
Polyporaceae	*G. frondosa*	CM756-2	ND
Schizophyllaceae	*S. commune*	CM557-2	ND
Strophariaceae	*H. sublateritium*	CM691-2	ND
	P. nameko	CM887-2	ND
	P. squarrosa	CM235-3	ND

[a] Each value represents the mean of three independent determinations. Figures express MPG-1 content (μg)/hot water extract (g) in dried weight. [b] ND, below the threshold of the assay.

Table 4. Temperature Stability of MPG-1 in *T. matsutake* Fruiting Bodies or Mycelial Preparation of Strain BP-7304 As Measured by a Sandwich ELISA

processing temperature	treating time	level of MPG-1[a] fruiting bodies	level of MPG-1[a] mycelia
	suspension condition		
control (25 °C)	control (3 h)	100.0	100.0
40 °C	3 h	98.9	98.4
60 °C	3 h	101.2	99.1
98 °C	3 h	99.7	104.3
121 °C	15 min	97.1	98.6
	dry condition		
control (25 °C)	control (8 h)	100.0	100.0
40 °C	0.5 h	100.3	100.4
	1 h	106.0	99.5
	2 h	97.6	102.9
	4 h	99.8	98.1
	8 h	102.2	100.4
60 °C	0.5 h	95.5	91.3
	1 h	108.6	99.4
	2 h	99.3	87.5
	4 h	92.1	84.9
	8 h	85.8	80.6
100 °C	0.5 h	91.8	85.9
	1 h	87.2	100.4
	2 h	95.6	89.2
	4 h	91.8	88.5
	8 h	70.5	76.2
150 °C	0.5 h	71.4	67.7
	1 h	38.5	47.6

[a] Values relative to control value (25 °C for 8 h) set to 100; each value represents the mean of three independent determinations.

Table 5. Detection of MPG-1 in Processed Foods Supplemented with *T. matsutake* Fruiting Bodies or Mycelial Preparation of Strain BP-7304

processed foods or drinks		supplementation of *T. matsutake*	MPG-1 content	recovery of MGP-1 (%)
soup	commercialized product	+ (fruiting bodies)	0.95 μg/mL[a]	
	commercialized product		ND[b]	
boiled rice	commercialized product	+ (fruiting bodies)	0.51 μg/g[c]	
	commercialized product		ND	
	homemade product	+ (mycelia)	68.8 μg/g[c]	21.0
	homemade product		ND	
breads	homemade product	+ (mycelia)	74.9 μg/g[c]	18.2
	homemade product		ND	
cookies	homemade product	+ (mycelia)	61.4 μg/g[c]	14.9
	homemade product		ND	
tea	homemade product	+ (mycelia)	40.2 μg/mL[a]	14.6
	homemade product		ND	
yogurt	homemade product	+ (mycelia)	58.8 μg/mL[a]	21.1
	homemade product		ND	

[a] MPG-1 content (μg/mL) of liquid materials. [b] ND, below the threshold of the assay. [c] MPG-1 content (μg/g) of dried material. Recovery of MPG-1 (%) was expressed as a percentage of recovered MPG-1 content to total MPG-1 content in fruiting bodies or mycelial preparation added to processed foods.

Next, the temperature stability of MPG-1 in dried powder form was examined. Dried powder of *T. matsutake* fruiting bodies or mycelial preparation was kept in an oil bath at 40, 60, 100, or 150 °C for 0.5, 1, 2, 4, or 8 h under a nitrogen atmosphere, and the MPG-1 content of the sample was determined using the ELISA system. As shown in **Table 4**, in samples derived from fruiting bodies and mycelial preparations exposed to 40 and 60 °C for 8 h, the levels of MPG-1 were 102.2 and 85.8 and 100.4 and 80.6, respectively, when setting the level for the 25 °C control group to 100. When samples were exposed to 100 °C for 8 h, the level gradually decreased with time, and the levels were 70.5 in the case of fruiting bodies and 76.2 in the case of mycelial preparation, respectively. Furthermore, when samples were exposed to a temperature of 150 °C for 1 h, the levels were markedly reduced to 38.5 in the case of fruiting bodies and 47.6 in the case of mycelial preparation, respectively (**Table 4**). The results suggested that the MPG-1 in dried fruiting bodies or mycelial preparation was relatively stable when exposed to temperatures below 100 °C for 4 h.

Consequently, it was examined whether MPG-1 in processed foods is detectable by the ELISA system. As shown in **Table 5**, the system was able to detect MPG-1 in all of the *T. matsutake*-supplemented processed foods, soup, boiled rice, bread, cookie, tea, and yogurt, and levels were below the detectable limit in control processed foods not supplemented with *T. matsutake*. These results suggest that the ELISA system is able to detect MPG-1 in processed foods.

T. matsutake is a unique ecomycorrhizal basidiomycete that occurs primarily within pine forests. It is harvested as a commercially valuable edible mushroom in East Asia including Japan, South Korea, North Korea, and China. It is also distributed in North America, North Africa including Morocco, and Europe including Sweden. The mycelia grow in symbiosis with Japanese red pine in the intercellular space of living root tips to form mycorrhizae, and fruiting bodies are produced during aggregative growth of mycorrhizas to develop a colony called "shiro". The annual yield of *T. matsutake* fruiting bodies has dramatically decreased since the 1940s in Japan. To prevent such decreases, various methods to improve production have been tried; however, much is still unknown about how the fruiting bodies form etc., and so, cultivation of the fruiting bodies of this species has yet to be achieved. Recently, a study at the gene level demonstrated considerable genetic diversification within the species, and DNA markers to discriminate and specify strains are being developed to select types suitable for the production and cultivation of fruiting bodies (*25*).

In the present study, we obtained a pAb that recognized the sugar moiety of MPG-1 sensitive to periodate treatment and distinguished it from 18 other polysaccharide preparations such as glycogen and starch (**Table 1**), although nine rounds of immunization in rabbits were necessary to attain an appreciable titer. Mekela et al. (*26*) reported that the characteristics of pAbs to sugar moieties were dependent on the antigenic nature of the sugar component and peptide component, ratio of sugar to protein, and molecular weight, when immunized with conjugate of dextran and BSA as a model compound. MPG-1 has an α-(1,4)-D-glucan as the main chain, with extensive branching by α-(1,2)- and α-(1,6)-D-linkages interacting with the protein. The ratio of sugar to protein is 95:5, the molecular mass is 360 kDa, and it is highly resistant to hydrolysis by amylase and protease (*17*). These factors might decide the nature of the pAb toward the sugar moiety in the MPG-1 molecule. Furthermore, antibody to β-D-glucan is reported to distinguish β-(1,3)-D-glucan with branches of β-(1,6)-D-glucan, which possesses antitumor activity, from other β-glucans including linear β-(1,3)-D-glucan, linear β-(1,6)-D-glucan, and β-(1,6)-D-glucan with branches of β-(1,4)-D-glucan (*27–29*). A mAb to β-D-glucan can distinguish linear β-(1,3)(1,4)-D-glucan with serum cholesterol-reducing activity from linear β-(1,4)-D-glucan and arabinoxylan in grain (*7*). According to the results of our preliminary experiments using fractions in the course of purification of MPG-1 from the mycelia (*17*), the pAb in the present study predominantly recognized MPG-1 or MPG-1-containing fractions and did not react, for example, with other inactive fractions such as EW-1, EW-3, EW-4, and EW-5. Furthermore, the pAb inhibited the in vitro activity of MPG-1 to bind with recombinant transforming growth factor-β_1. Thus, immunological detection methods utilizing specific pAbs are suitable for the discrimination of high molecular weight substances with a complicated stereostructure such as MPG-1 and β-D-glucan, from multiple components with similar structures. Moreover, concerning immunomodulatory α-D-glucans, this is the first report that an ELISA system using a pAb can distinguish them from polysaccharides in mushrooms.

The results of screening the extracts of mushrooms with the ELISA system suggested that the molecule was detected predominantly in *T. matsutake* in not only fruiting bodies but also mycelia, and it was below the detection limit in three species belonging to the genus *Tricholoma* in Tricholomataceae, six species belonging to other genera in Tricholomataceae, and 25 species in 12 families other than Tricholomataceae. There are a few characteristic physiologically active agents in mushrooms including *T. matsutake*; alkaloids such as muscarine (*30*),

amino acids such as tricholomic acid and ibotenic acid (*31*), and volatile compounds such as 1-octen-3-ol (*32*) have been identified. The antitumor protein reported by Kawamura et al. (*33*) was found predominantly in fruiting bodies of *T. matsutake* and was not present in cultured mycelia. The nature of these agents is clearly different from that of MPG-1.

The content of MPG-1 in *T. matsutake* fruiting bodies varied according to the sampling area or site. It is speculated that differences in content among sampling areas are based mainly on the sensitivity of strains to the environmental factors such as the soil components or climate or the capacity of strains to produce polysaccharides. To minimize the effects of environmental factors, as in the case of the fruiting bodies, when cultured mycelia were examined as starting materials, MPG-1 was detected predominantly in *T. matsutake* species. Notably, the MPG-1 content of *T. matsutake* strain BP-7304, which was isolated from the fruiting bodies sampled at Kyoto, was superior to that of eight standard strains, suggesting that there were strain differences in the productivity. Thus, the present study confirmed that *T. matsutake* is the fungus that predominantly produces MPG-1.

The antigenicity of MPG-1 was little affected when the water suspension or dried powder of *T. matsutake* was kept below 100 °C for 4 h or at a pH of between 3.0 and 8.0 for 3 h, suggesting that its conformation was relatively stable. According to the results of the thermal analysis, the dried powder of fruiting bodies or mycelia of *T. matsutake* showed a start temperature of exothermic peak under a nitrogen atmosphere of 234 and 272 °C as measured by differential scanning calorimetry and a start temperature of heat decomposition of 187 and 215 °C as measured by thermogravimeter, respectively, which supported the above results regarding the stability of the structure of MPG-1 (unpublished data). Furthermore, MPG-1 was detected not only in the commercialized products of mushroom-processed foods but also in homemade mushroom-processed foods, such as soup, boiled rice, bread, cookie, and yogurt, without affecting the quality of foods such as taste, aroma, and outward appearance. Thus, the system in the present study is considered of value for not only the characteristic analysis of MPG-1 but also the development and quality control of mushroom-supplemented processed foods or supplements.

Finally, we confirmed that the ELISA system established in this study is sensitive and selective and is relatively simple to operate and to measure multisamples, as compared to the conventional methods of sugar analysis methods such as AOAC (*18*) and microelectrophoresis methods (*19*). Some kinds of edible mushrooms have been recognized by the people of not only East Asia but also Europe and North America as healthy foods low in calories and rich in vitamins, minerals, and dietary fibers. The characterization of physiologically active α-D-glucan in the present and previous studies (*17*) is of value. Further evaluation for applications in the fields of processed foods and medical products is under investigation.

NOTE ADDED AFTER ASAP PUBLICATION

The original posting of September 15, 2007, contained an incorrect sugar linkage for lentinan in Table 1. This has been corrected with the posting of September 20, 2007.

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